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The etiology of prostate cancer is not well understood. Exposure to carcinogenic heterocyclic amines such as PhIP, formed in high-temperature cooked meat has been implicated as an important risk factor. The Phase I metabolic pathway of PhIP is mainly catalyzed by the human cytochrome P450s 1A2, 1A1, and 1B1 to form 2-hydroxyamino-PhIP (*N*-hydroxy PhIP), the carcinogenic metabolite, and 4'-hydroxy PhIP (4'-hydroxy PhIP), the non-carcinogenic metabolite. Although epidemiological studies suggest that PhIP may play an important role in the etiology of human colon, breast, or prostate cancer, the capability of these human extrahepatic tissues in metabolizing PhIP has not been well studied. In the present study, metabolic activation of food borne heterocyclic amines in 31 different human prostate microsomes indicates that there is no significant *N*-hydroxylation of PhIP, IQ, MeIQ and MeIQx.

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## **Introduction** (modified from previous report)

In the United States, prostate cancer is the most commonly diagnosed cancer in men, and the incidence is rising rapidly. According to a projection by the American Cancer Society, a total of 230,110 new cases of prostate cancer are estimated in the United States in the year 2004 and About 29,900 men will die of this disease. They also estimate that African-American men are more likely to have prostate cancer and to die of it than are white or Asian men and the reasons for this are still not known (1) [www.cancer.org](http://www.cancer.org). According to a projection by the Prostate Cancer Charity organization in UK, total of 542,909 new cases of prostate cancer are diagnosed globally and about 204,313 men will die of prostate cancer <http://www.prostate-cancer.org.uk/learn/bigPicture/index.asp>. Despite extensive efforts, the origins of prostate cancer and the factors that promote its progression are not well established; however, epidemiological evidence shows strong associations with dietary fat intake and red meat consumption (2,3).

The majority of the mutagenicity in meat cooked under normal household conditions may be accounted for by a single group of heterocyclic amines (HCAs) such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*] quinoline (MeIQ) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (Fig. 1) (4). The positive correlation between meat consumption and cancer incidence such as those of the stomach (5), prostate (6), breast (7,8), lung (9) in human populations (10) along with carcinogenicity to rats and mice (11-13) of these compounds suggest that they may be an important factor in diet-related human cancers. The critical step in HCA bioactivation is catalyzed by the CYP mediated *N*-hydroxylation, forming its major metabolite, *N*-hydroxy HCA (14-17). The presence of CYP1A2, CYP1A1 and CYP1B1 mRNA in the human prostate has been reported (18). Chemical toxicity and carcinogenicity in extrahepatic tissues are frequently results from in situ metabolic activation mediated by the target organ and the toxicity of a given compound is linked to its metabolism in the target tissue (19-21). Prostate tissues obtained from rats fed with HCAs, were histopathologically evaluated and found to contain prostate carcinomas (22, 23). Risk assessment of potential human carcinogens based primarily on data obtained from experimental animals. Due to the species differences in metabolic activation, a thorough characterization of the expressed CYP enzymes in human prostate tissues is crucial for a more accurate prediction of prostate cancer risk. In the present work, we examined 31 different human prostate tissues for their metabolic capacity to activate PhIP and other carcinogenic HCAs.

### *The specific aims are adopted from the grant application*

1. To confirm our preliminary finding that a large interindividual variation exists in the PhIP-metabolizing activity of the human prostate with a large number of samples (~100 samples), and to examine the existence of such a variation in the metabolism of other carcinogenic HCAs such as IQ, MeIQ, and MeIQx found in well-done meat. For the metabolic activation study, our newly developed LC/ESI-ITMS method will be used.
2. To determine the relationship between the individual variation in prostate HCA-metabolizing activity and the mRNA/protein expression profiles of HCA-metabolizing CYP enzymes (CYP1A2, CYP1A1, and CYP1B1) in the prostate by correlation analysis. Quantitative real-time RT-PCR and immunoblots analyses will be used for measuring the mRNA and protein levels of the CYPs involved.
3. To investigate the functional significance of the genetic variants of the human CYP enzymes that are responsible for the metabolism of PhIP and other HCAs in human prostate. We will determine the distribution of known functional genetic polymorphisms of the HCA-metabolizing CYPs and correlate the genotype with the phenotype (HCA-metabolizing activity and CYP mRNA/protein levels in the prostate) of the same individual. We will also screen for novel genetic polymorphisms of CYP1A2, CYP1A1, and CYP1B1 in the samples having either very high or low HCA-metabolizing activity. Once such polymorphic variants are identified, we will conduct functional characterization study. For this part of the work, we will apply most of the approaches and methods that have been successfully used in our current study of CYP2A6 genetic polymorphism.

## **Body**

### *Materials*

PhIP, IQ, MeIQ, MeIQx, *N*-hydroxy PhIP, *N*-hydroxy IQ, *N*-hydroxy MeIQ were purchased from the Toronto Research Chemicals Canada, NADPH was obtained from Sigma Chemical Co. (St. Louis, MO). The protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA). HPLC grade solvents were purchased from Burdick and Jackson (Muskegon, MI). 4-hydroxy PhIP was a generous gift from Dr. Kulp and KS, Dr. Knize MG, Lawrence Livermore National Laboratory (LLNL), Livermore, CA and Dr. Wakabayashi laboratory, NCCRI, Tokyo, Japan. Other reagents were of the highest grades available commercially. PTFE-membrane syringe filters were from Sartorius AG, Germany. Recombinant cDNA-expressed human CYP1A1, CYP1A2 and CYP1B1 and human liver microsomes were obtained from Gentest, Woburn, MA.

### *Human Prostate tissues*

All samples were snap-frozen within 1-hour post surgery. Samples were obtained at the time of surgery, immediately frozen in liquid nitrogen and shipped in a dry ice container. Prostate tissues were stored at -70°C until the preparation of microsomal fractions. Human prostate tissues were obtained from the Cooperative Human Tissue Network (CHTN), (Philadelphia, PA) and National Disease Research Interchange (NDRI, Philadelphia, PA) after prior approval of the Institutional Review Board of University of Medicine and Dentistry of New Jersey. The details of the subjects for the prostate tissue are in Table 1.

### *Human prostate microsome preparation*

Prostate microsomes were prepared after a small modification as described in (25-27). The tissues were collected from 31 different individuals undergoing the prostate surgery (Table 1). The frozen tissues were briefly thawed to 4°C and a small portion of the tissue was cut and stored back in -70°C for the RNA studies. The remaining tissue was weighed and homogenized for three 15 seconds intervals with a Polytron steel blade homogenizer followed by a three complete strokes in a Potter-Elvehjem homogenizer in a 250 mM sucrose containing 5 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10 mM KCl; 0.1 mM EDTA, 1 mM MgCl<sub>2</sub> pH 7.2 and 0.1 mM phenylmethyl sulfonyl fluoride, 1mM dithiothreitol was added just before the preparation. The homogenate was subjected to centrifugation at 9000 g for 20 min and the resulting pellet was discarded. The supernatant fractions were subjected to centrifugation at 100,000 g for 90 min. The microsomal pellet was suspended in 250 mM sucrose (pH 7.2 Tris buffer) as concentrated suspension and stored in -70°C. All procedures were essentially carried out at 2 to 4°C. Protein estimation and enzyme assay was conducted immediately.

### *Enzyme assay conditions*

A typical incubation mixture (0.23 ml final volume) consisted of 100 mM sodium phosphate buffer (pH 7.4), prostate microsomal proteins (0.6 – 1 mg/ml), and 200 µM of the HCA substrate (either of PhIP, IQ, MeIQ, MeIQx). The reaction was initiated by the addition of the 1 mM NADPH after 3 min preincubation at 37°C and terminated at the end of 30 min by the addition of an equal volume of ice-cold methanol. After a brief vortex mixing and centrifugation at 4°C for 30 min the supernatant was filtered using PTFE 0.4 µm syringe filters. Negative control experiments consisted of incubation of HCA with liver and prostate microsomes inactivated by addition of ice-cold methanol prior to incubation. For positive control, liver and human recombinant microsomes were used.

### *Analytical conditions*

Analysis was performed on a HPLC system comprising of a Spectra P4000 separation module consisting a quaternary pump equipped with a Spectra UV600LP photodiode array UV detector, auto-sampler (AS 3000) maintained at 2°C and Thermo Finnigan LCQ Decca mass spectrometer (Thermo, San Jose, CA). The mass spectrometer equipped with an electrospray ionization (ESI) source and an ion trap mass analyzer (IT). The LC flow was introduced into the ESI following the detection by UV



absorption from 200 to 400 nm. The chromatography was performed on a Symmetry C<sub>18</sub> reversed-phase column 2.1 x 100 mm column, Waters, (Milford, MA). The analytical column was protected by a C<sub>18</sub> Security Guard system from Phenomenex (Torrance, CA). The separation of HCAs and metabolites were carried out using a isocratic elution program of a binary mobile phase at a flow-rate 100 µl/min composed by 0.5 mM ammonium acetate buffer containing 19% methanol, 0.1% acetic acid and 0.5% tetrahydrofuran (Solvent A), 70% Methanol (Solvent B) and 0.1% acetic acid, 0.1% methanol in 5mM ammonium acetate (Solvent C). In the analysis of PhIP and *N*-hydroxy PhIP 50:50 ratio of A:B were used and for the analysis of IQ, MeIQ, MeIQx and their hydroxy metabolites 70:30 of A:C were used. In the analysis of 4-hydroxy PhIP the mass spectrometer having dual segments in single reaction monitoring (SRM) or a full scan multiple reactions monitoring (MRM) of the parent ion *m/z*=241 with the range of collision energy from 30% - 45% in the IT reduced the detection sensitivity at low level detection of *N*-hydroxy PhIP. Also the relative detector response for 4'-hydroxy PhIP was much higher than the *N*-hydroxy PhIP and the 4'-hydroxy PhIP did not form fragments with 30% collision energy, hence these to metabolites were selectively analyzed separately in SRM mode for *N*-hydroxy PhIP and 4'-hydroxy PhIP.

The voltage on the ESI interface was maintained at +5 kV in the positive ion mode. High-purity nitrogen gas served both as sheath gas with an operating pressure of 90 psi and as auxiliary gas with a flow-rate of 2.6 arb. The heated capillary temperature and voltage were maintained at 250°C and 3.1 V, respectively. The tube lens offset voltage was set at -16.2 V. The ion trap was operated in selected ion monitoring (SIM) (MS) mode and selected reaction monitoring (SRM) (MS/MS) mode. To prevent MS contamination when running MS, a divert valve was used, the first 4 minutes fractions from the column was sent to waste. A solution of caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate, and Ultramark 1621 prepared in mixture of acetonitrile : methanol : water (2:1:1) containing 1% acetic acid was used for the calibration of mass spectrometer. Data acquisition and system control was performed using Xcalibur software.

### *Experimental Design*

The overall tasks involved in this part of the study were designed and divided into four parts:

- *enzyme assay and sample preparation*
- *determination of selective MS conditions*
- *sample analysis*
- *data processing and review*

### *Enzyme assay and Sample Preparation*

Due to insufficient amount of the prostate tissues and microsomes, we have used different amount of human liver microsomes to optimize the PhIP metabolism incubation conditions. With a low amount of human liver microsomal protein (5 µg) for the incubation at different time points to determine the linear range of the metabolite formation. The *N*-hydroxy PhIP metabolite formation was linear up to 45 min (Figure 3) based on this results all the prostate microsomes were incubated for 30 min.

Until now, we have collected 31 human prostate tissues and prepared the microsomes from all of them. These prostate microsome samples were incubated with PhIP for 30 min along with the negative control and liver microsomes, human recombinant enzymes CYP1A1, CYP1A2 and CYP1B1 as positive controls. To determine the concentrations of the metabolites formed, known amounts of *N*-hydroxy PhIP was added to the incubation mixture (containing pre-inactivated microsomes) and the authentic standard *N*-hydroxy PhIP was analyzed by following the same protocols as for the samples. Similarly, under identical conditions other HCAs such as IQ, MeIQ and MeIQx were also incubated for 30 minutes.

### Determination of Selective MS Conditions

In addition to the carcinogenic metabolite *N*-hydroxy PhIP, microsomal incubation system may generate other isomeric and oxidative metabolites such as 2-hydroxy PhIP, 4'-hydroxy PhIP, and 5-hydroxy PhIP. It is necessary to validate selectively the metabolites of interest from such complex isomeric mixtures to avoid interference during the quantitation. The ion trap was operated in selected ion monitoring (SIM) mode and selected reaction monitoring (SRM) mode. The first injection was SIM or SRM 0% collision energy in the positive ion mode to determine the optimal precursor ion. The second injection alternatively switches between several collision energies to determine the optimal product ion and collision energy for each precursor ion found from the first injection. Finer optimization of the collision energy was accomplished manually by incorporating additional collision energy to the computer-generated value.

Highly sensitive and selective LCMS and MSMS methods were developed for the analysis of PhIP, IQ, MeIQ, MeIQx and their respective *N*-hydroxy metabolite. Our recently developed direct injection method avoids the "solid phase extraction" and other lengthy sample preparation steps and includes a simple online sample clean up step.

### Sample Analysis

After sample preparation the incubation mixture was loaded on HPLC column through the auto sampler (with a constant injection volume of 20  $\mu$ l), the column eluent containing phosphates and salts from the incubation medium is directed to waste for a short time 3-4 min, after which the valve switches and the mobile phase directed through the column, causing the analyte elution and goes for the mass spectrometric detection. In the present method, the chromatography system serves to desalt the sample. For reliable and reproducible quantitation, at the column dimension and flow rates used it is necessary to flush and recondition the column after injection of every 5 samples in order to maintain the column in ideal condition. Additionally, standards samples and quality control (QC) samples were injected in the beginning and end of each run as well as intermittently. The precision and accuracy of the assay were determined by replicate analyses of QC samples.

Calibration standards were prepared in triplicates (1 to 50 ng/ml) (Fig. 2). The assay was validated for reliable, repeatable quantitation of *N*-hydroxy PhIP and the linearity of ion-trap response. The *N*-hydroxy metabolite formation was comfortably quantified (Fig. 5) in PhIP incubation containing 1  $\mu$ g of human liver microsomal protein (0.004 mg/ml final protein concentration). The assay takes <15 min with isocratic elution including the online sample clean up step and suitable for high throughput analysis. This method provides good separation of the substrate and the metabolite to selectively quantitate the *N*-hydroxy PhIP from the incubation system and the lower limit of quantification (LLOQ) is 20 pg. The lowest limit of detection of the assay was the lowest amount the calibration curve, with acceptable precision (20%) and accuracy ( $100 \pm 10\%$ ). The quality control (QC) samples were prepared in the same fashion as the calibration samples at concentrations corresponding to 1 and 10 ng/ml. A blank sample (no standard) was prepared by mixing 50:50 ratios of the mobile phase. The stability and reliability of the assay were determined by replicate analyses of QC samples. The LC-MS system was routinely checked for carryover by injecting the blank after every 5 samples and also after the injection of high concentration *N*-OH-PhIP.

The peaks were identified based on their retention time in the HPLC-UV chromatogram and their UV spectrum, which was performed by the human recombinant CYP1A2 (Fig. 4a) and their molecular weight precursor ion in the LC/MS mode. To further confirm the identity of *N*-hydroxy PhIP ( $m/z$  241), the peak obtained from the incubation system was subjected to fragmentation in MS/MS mode by 30% collision energy. The base peak in the MS/MS spectrum for *N*-hydroxy PhIP ( $m/z$  224) originated from the loss of  $[M+H-OH]^+$  from the *N*-hydroxy PhIP molecular ion ( $m/z$  241). A similar base peak fragment was observed in the MS/MS spectrum from the authentic standard *N*-hydroxy PhIP. Similarly the, UV, MS and MSMS fragments for *N*-hydroxy IQ, (Fig. 4b)  $[M+H]$   $m/z$  215 and  $[M+H-OH]^+$   $m/z$  198, *N*-hydroxy MeIQ (Fig. 4c)  $[M+H]$   $m/z$  229 and  $[M+H-OH]^+$   $m/z$  212 and *N*-hydroxy

MeIQx (Fig. 4d)  $[M+H]$   $m/z$  230 and  $[M+H-OH]^+$   $m/z$  213 were obtained. The authentic standards of *N*-hydroxy IQ and *N*-hydroxy MeIQ were highly unstable and could not be used for quantitation. In case of 4-hydroxy PhIP  $m/z$  241 formed from the incubation system subjected to fragmentation in MS/MS mode by 40% collision energy. The base peak in the MS/MS spectrum for 4-hydroxy PhIP ( $m/z$  226) originated from the loss of  $[M+H-CH_3]^+$ . The formation of 4-hydroxy PhIP was qualitatively confirmed by the standard samples and the formation of 4-hydroxy PhIP was consistent as reported by Hammons *et. al*, (23), the formation of 4-hydroxy PhIP by human CYP1A1 was shown in Fig. 8.

#### Data Processing and Review

It takes significant amount of time, after analysis of a sample set each raw file must be opened for review and processing of the data. Xcalibur software (San Jose, CA) was used to process the data and extract the peak area and height into a spreadsheet.

#### Results

*N*-hydroxylated metabolites of PhIP, IQ, MeIQ and MeIQx were detected in the reactions with 5  $\mu$ g (0.02 mg/ml) human liver microsomes, CYP1A1, CYP1A2 and CYP1B1 but not in the negative controls with the pre-inactivated microsomes or incubation without NADPH. Substantial amount of *N*-hydroxy PhIP, *N*-hydroxy IQ, *N*-hydroxy MeIQ, and *N*-hydroxy MeIQx formation was detected in human liver microsomes and in the human recombinant enzymes (Fig. 4). In case of the liver microsomes, the rate of *N*-hydroxy PhIP formation was found to be  $150 \pm 29$  pmol/min/mg protein. When the metabolic activity PhIP was assayed for prostate microsomes the activity was not detectable in 29 human subjects and very low formation of *N*-hydroxy PhIP was detected in two of the subjects P7 and P22 (Figure 6, 7 and 10). In prostate microsome the rate of *N*-hydroxylation was found to be  $0.28 \pm 0.05$  pmol/min/mg protein, about 800 times lower than the liver. The 4'-OH PhIP was also formed in this subject (Fig. 9a) and the formation was not detected in negative control (Fig. 9b); even using high protein concentration (1 mg/ml) and longer incubation time (60 min) the *N*-hydroxylation was not detectable in rest of the subjects. When the metabolic activity of IQ, MeIQ and MeIQx were assayed for prostate microsomes the *N*-hydroxyl metabolite was not detected in all the prostate microsomes.

Since the tissue P7 and P22 were obtained from normal prostate tissue and the remaining tissues were obtained from Benign Prostatic Hyperplasia (BPH) patients (Table 1). The results indicate that the normal prostate microsomes might have very low metabolic activity. However, it is difficult to assess the extent to which *in vitro* data reflect activities in intact prostate gland *in vivo*. This observation needs to be confirmed using a larger number of normal prostate tissue samples and not BPH tissues (studies ongoing). In summary our *in vitro* results indicate that human prostate microsomes do not metabolize the HCAs investigated to any greater extent.

#### Difficulties

LC/MS instrument is being used from the core facility of EOHSI analytical center, which is shared by an increasing number of users. The time limit in using the instrument affects the efficiency of our analysis.

The *N*-hydroxy IQ and *N*-hydroxy MeIQ standards (purchased from Toronto Research Chemicals Inc., Canada) we received were already decomposed. The manufacturer confirmed that the stock at their facility had also decomposed and agreed to pay a full refund or replace with freshly prepared compound.

The HPLC analytical column had to be replaced due to its age and inconsistency. A new HPLC analytical column with a shorter ID (2.1) to improve performance was purchased. However, the newly purchased column started malfunctioning and eventually became unusable and the column was replaced again and the conditions were re-optimized.



We have moved our laboratory from the EOHSI building to the new laboratory at School of Public Health Building.

Procuring the surgical human prostate tissue in a workable quantity is difficult. We are in the process of collecting more prostate samples in order to reach our goal, which is to collect  $\approx 100$  prostate tissues.

The reference standards of the metabolites *N*-hydroxy MeIQ, *N*-hydroxy IQ and *N*-hydroxy MeIQx are not available due to its instability. The differences are attributed largely to the structure and ionization efficiency differences between analytes. The metabolite with OH- moiety has much lower stability than the substrate. As a provision, while reference standards are not readily available, qualitative analysis of metabolite level was done based on the structurally related analog (substrate) with comparable ionization efficiency. The 4-hydroxy PhIP standard obtained from LLNL and NCCRI were not suitable low-level metabolite quantitation and were used as qualitative reference.

Even though we have identified and do our best to solve these problems in a timely manner, the crisis has affected our schedule.

### Key Research Accomplishments

- The LC/MS method has been further improved, validated and modified to a highly sensitive and selective LC-MS/MS method for the analysis of PhIP, IQ, MeIQ, MeIQx and their *N*-hydroxy metabolites.
- The *N*-hydroxy metabolite formation was selectively determined in the incubation system containing 0.004 mg/ml and 0.02 mg/ml of liver microsomal protein.
- Completed the study on metabolic activation of PhIP with 31 human prostate tissues. This the first report on HCA metabolism in human prostate microsome with relatively good sample size.
- Microsomes prepared from BPH prostate tissues or enlarged prostate tissue might not reflect the actual enzyme activity in normal prostate tissue.

### Reportable outcomes

None, A manuscript summarizes the metabolism of PhIP and other HCA by human prostate microsome is in preparation.

### Conclusion

Dietary HCAs is associated with an increased risk of prostate cancer through production of carcinogenic compounds that require biotransformation by enzymes. The roles of polymorphic cytochrome P450 (CYP) enzymes in HCA metabolism *in situ* are of particular interest in prostate cancer. The result presented in this study demonstrates that in the BPH, human prostate tissue does not metabolize the HCAs investigated to any greater extent. The results suggest that there is no *in situ* bioactivation of HCAs in the prostate tissue. The reported association between HCAs and prostate cancer in animal studies could be due to the hydroxy metabolites of HCAs, which are activated by the CYPs in the liver and transported to prostate. We will continue to collect more normal prostate tissue and extend the study on the metabolic activation of PhIP and other HCAs. We will also initiate the experiments proposed for Specific Aim 2 and 3.

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## Appendices

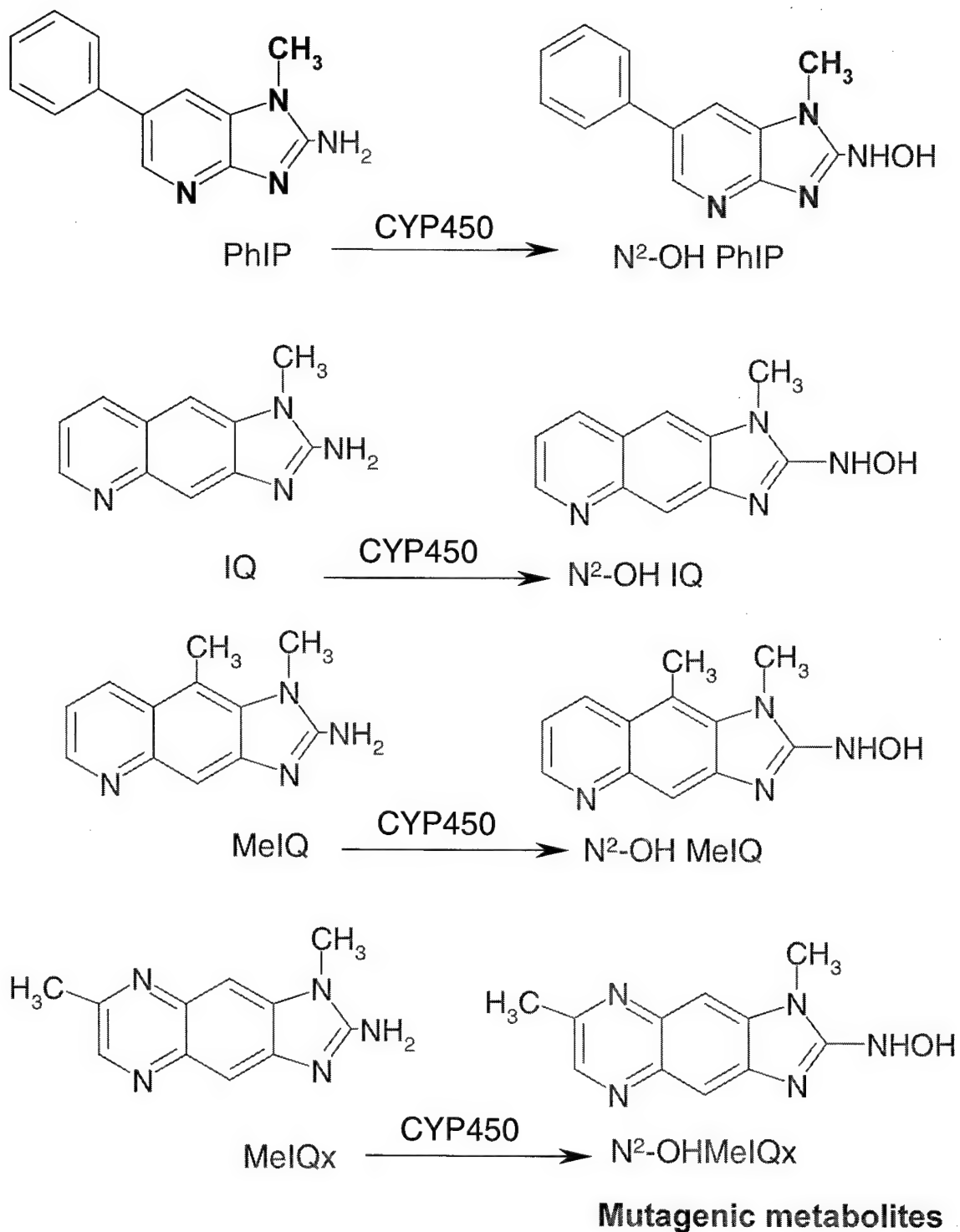


Figure 1: Chemical structure of the heterocyclic amines and its mutagenic metabolites

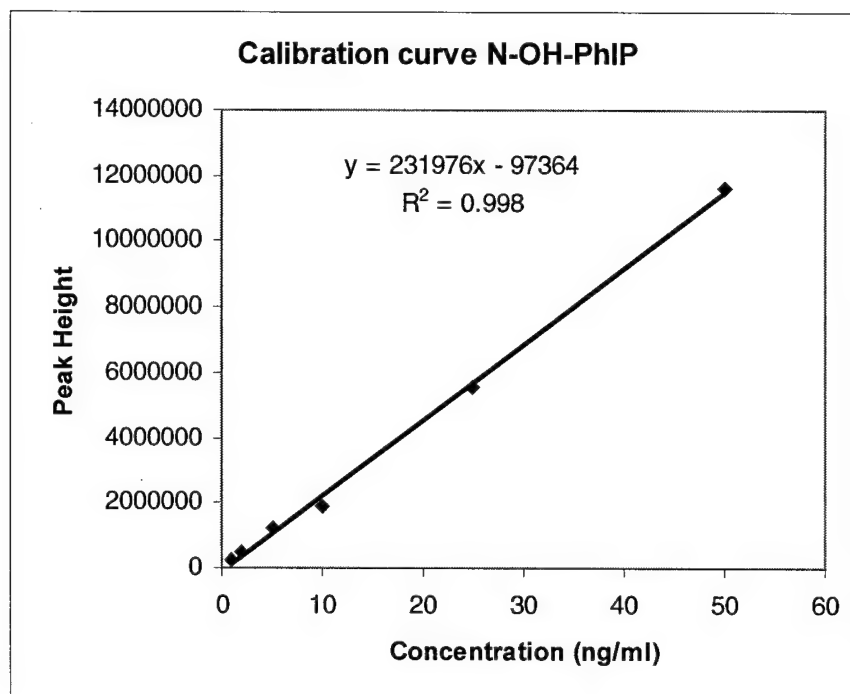


Figure 2: Calibration curve for *N*-hydroxy PhIP (1 ng/ml to 50 ng/ml standards were spiked into the incubation mixture containing inactivated human liver microsomes and the mixture was processed as described in sample preparation, constant volume 20  $\mu$ l was injected for the analysis).



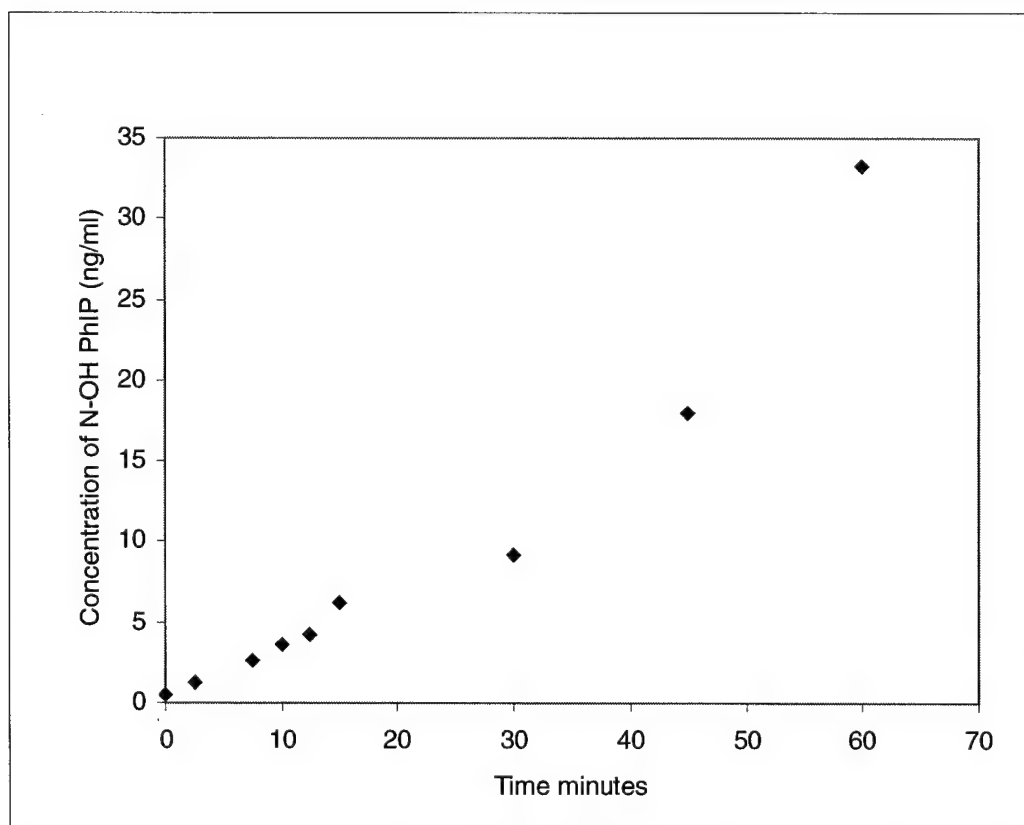


Figure 3: Formation of *N*-hydroxy PhIP vs incubation time, PhIP incubated with 5  $\mu$ g human liver microsome proteins for 2.5, 5, 7.5, 10, 12.5, 15, 30, 45 and 60 minutes. The *N*-hydroxy PhIP formation was linear up to 45 min and the incubations in prostate microsomes were done for 30 minutes.

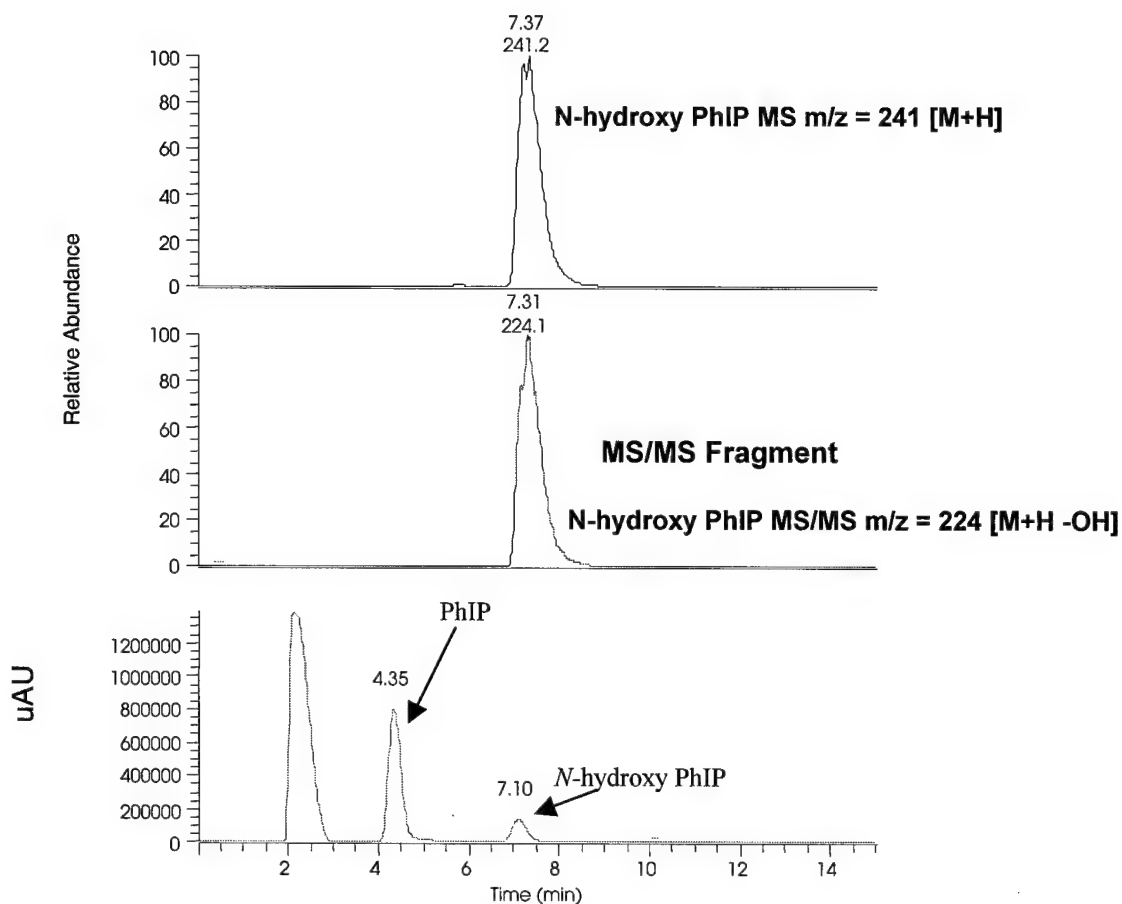


Figure 4a: HPLC-UV, MS and MS/MS analysis of PhIP and *N*-hydroxy PhIP, metabolism of PhIP by 5 pmol of human recombinant CYP1A2 incubated with 200  $\mu$ M PhIP for 30 min. Similarly the *N*-hydroxy PhIP formation was detected in human recombinant CYP1A1, CYP1B1 and human liver microsomes.

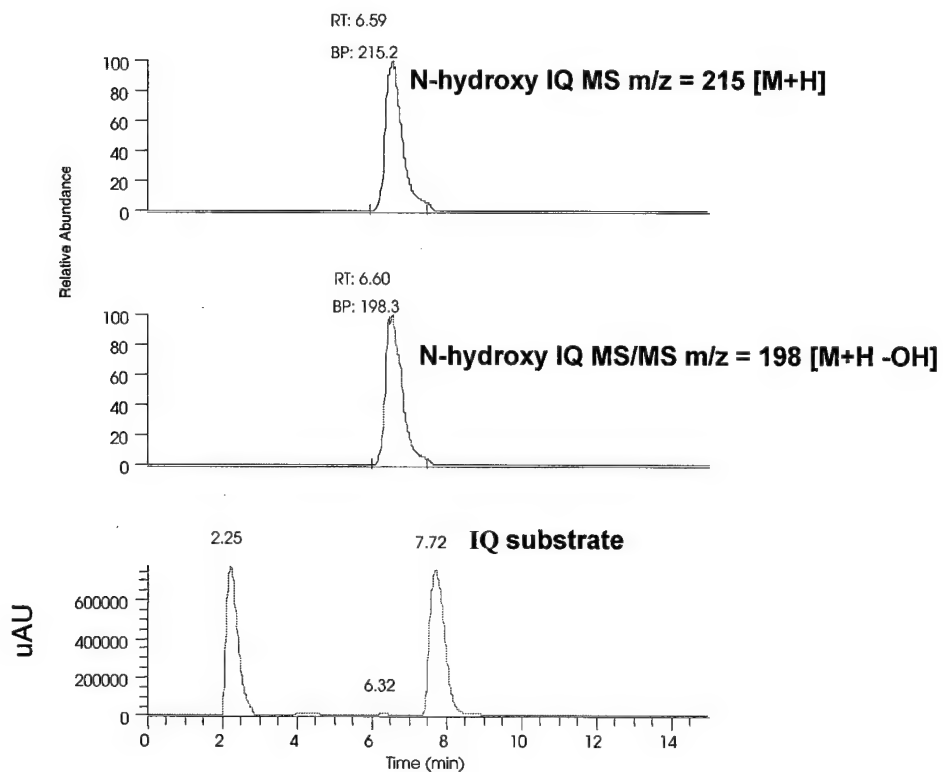


Figure 4b: HPLC-UV, MS and MS/MS analysis of IQ and *N*-hydroxy IQ, metabolism of IQ by 5 pmol of human recombinant CYP1A2 incubated with 200  $\mu$ M IQ for 30 min. Similarly the *N*-hydroxy IQ formation was detected in human recombinant CYP1A1, CYP1B1 and human liver microsomes at a lower level.

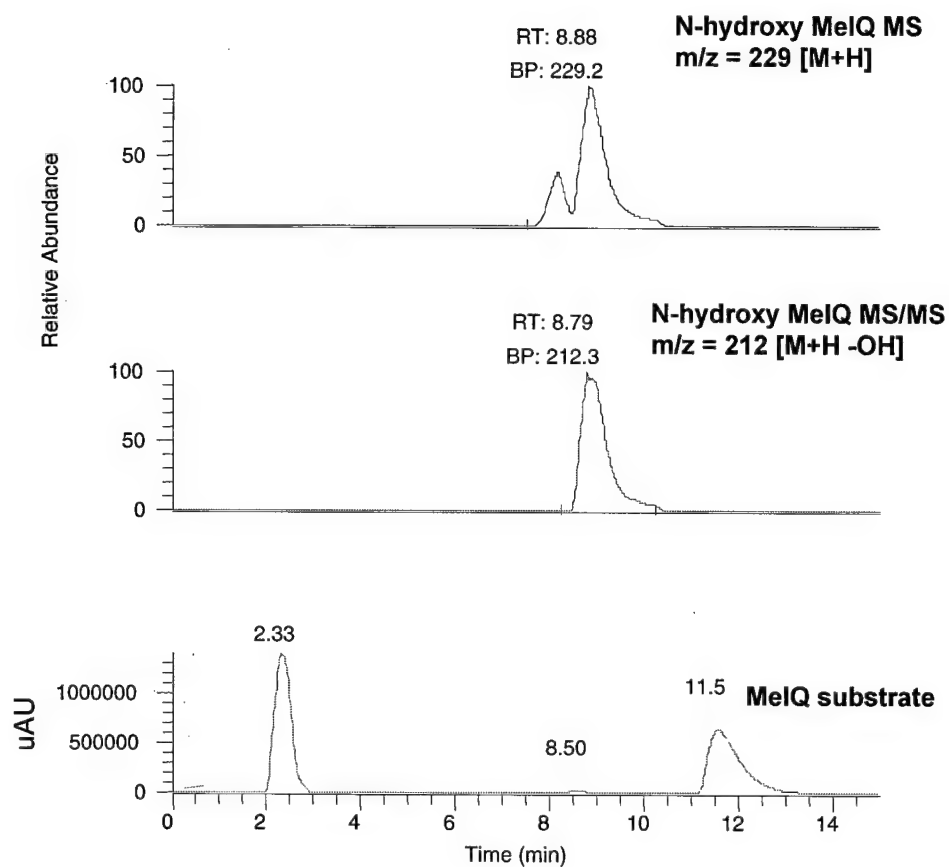


Figure 4c : HPLC-UV, MS and MS/MS analysis of MelQ and *N*-hydroxy MelQ, metabolism of MelQ by 5 pmol of human recombinant CYP1A2 incubated with 200  $\mu$ M MelQ for 30 min. Similarly the *N*-hydroxy MelQ formation was detected in human recombinant CYP1A1, CYP1B1 and human liver microsomes at a lower level.

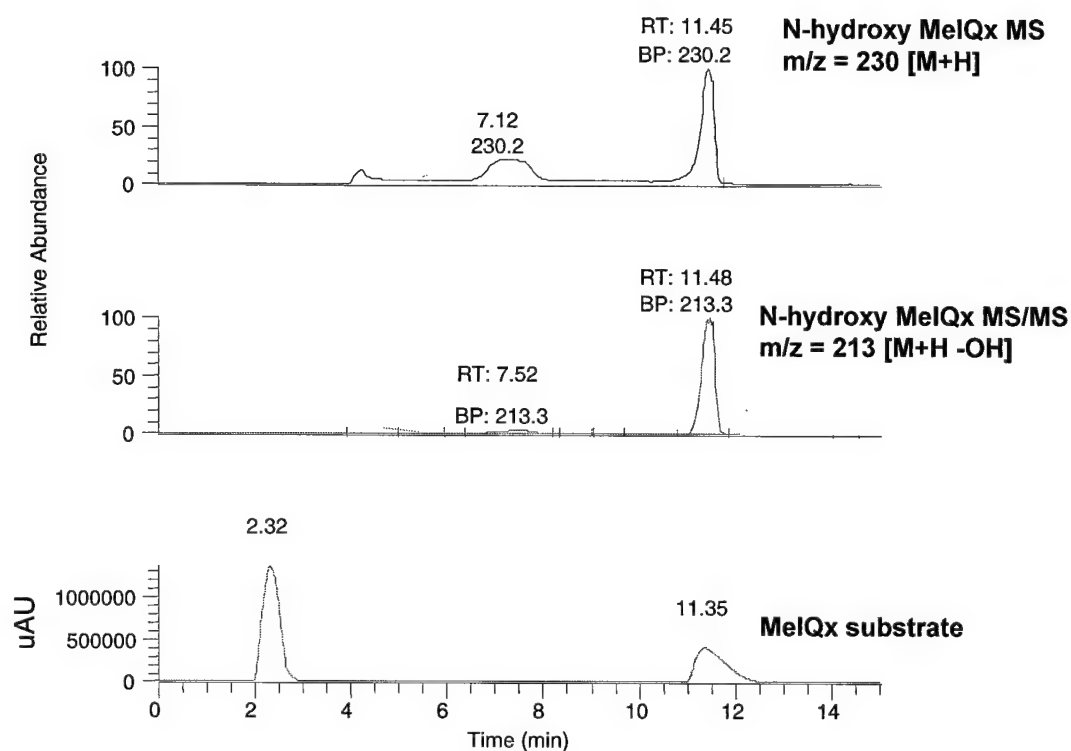


Figure 4d: HPLC-UV, MS and MS/MS analysis of MelQx and *N*-hydroxy MelQx, metabolism of MelQx by 5 pmol of human recombinant CYP1A2 incubated with 200  $\mu$ M MelQx for 30 min. Similarly the *N*-hydroxy MelQx formation was detected in human recombinant CYP1A1, CYP1B1 at very low level as compared to IQ and MelQ. The *N*-hydroxy MelQx formation was not detectable at 5  $\mu$ g human liver microsomes and comfortably detected at 50  $\mu$ g human liver microsomes.



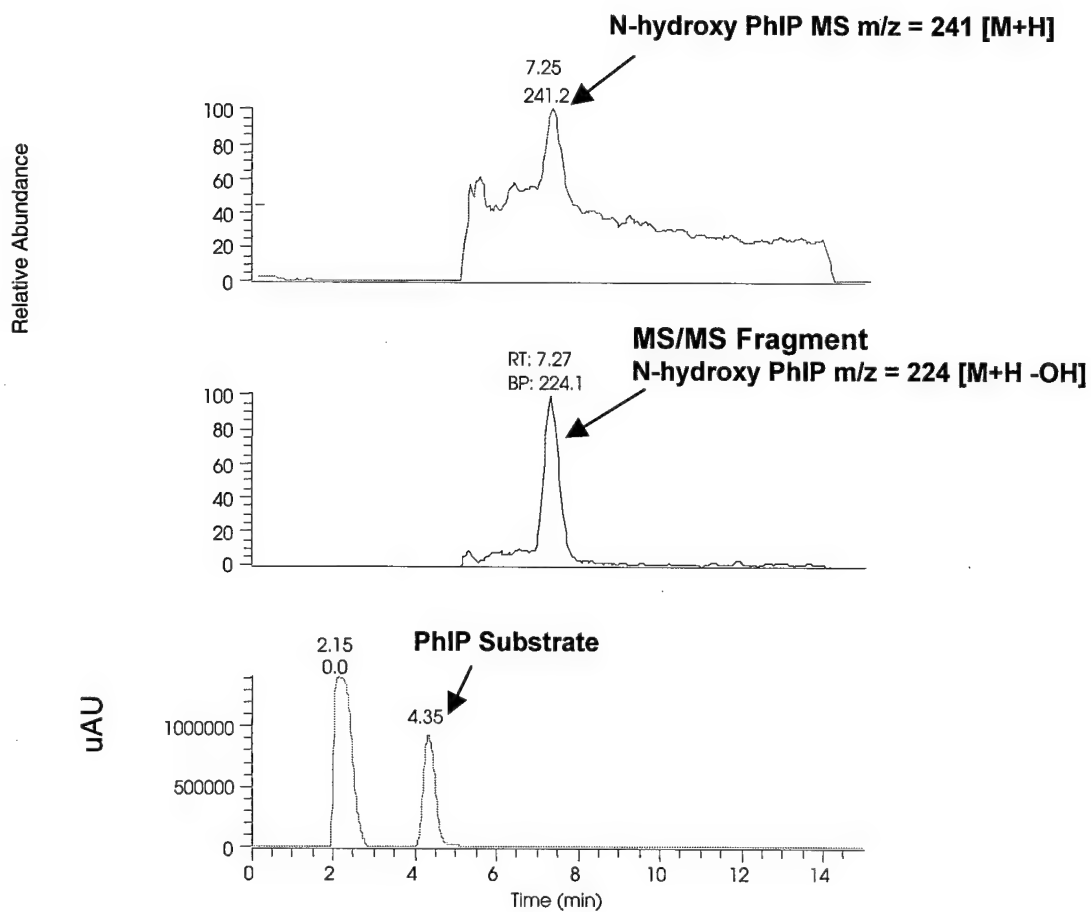


Figure 5: HPLC-UV, MS and MS/MS analysis of PhIP and N-hydroxy PhIP, metabolism of PhIP by human liver microsomes (1  $\mu$ g protein) incubated with 200  $\mu$ M of PhIP for 30 min.

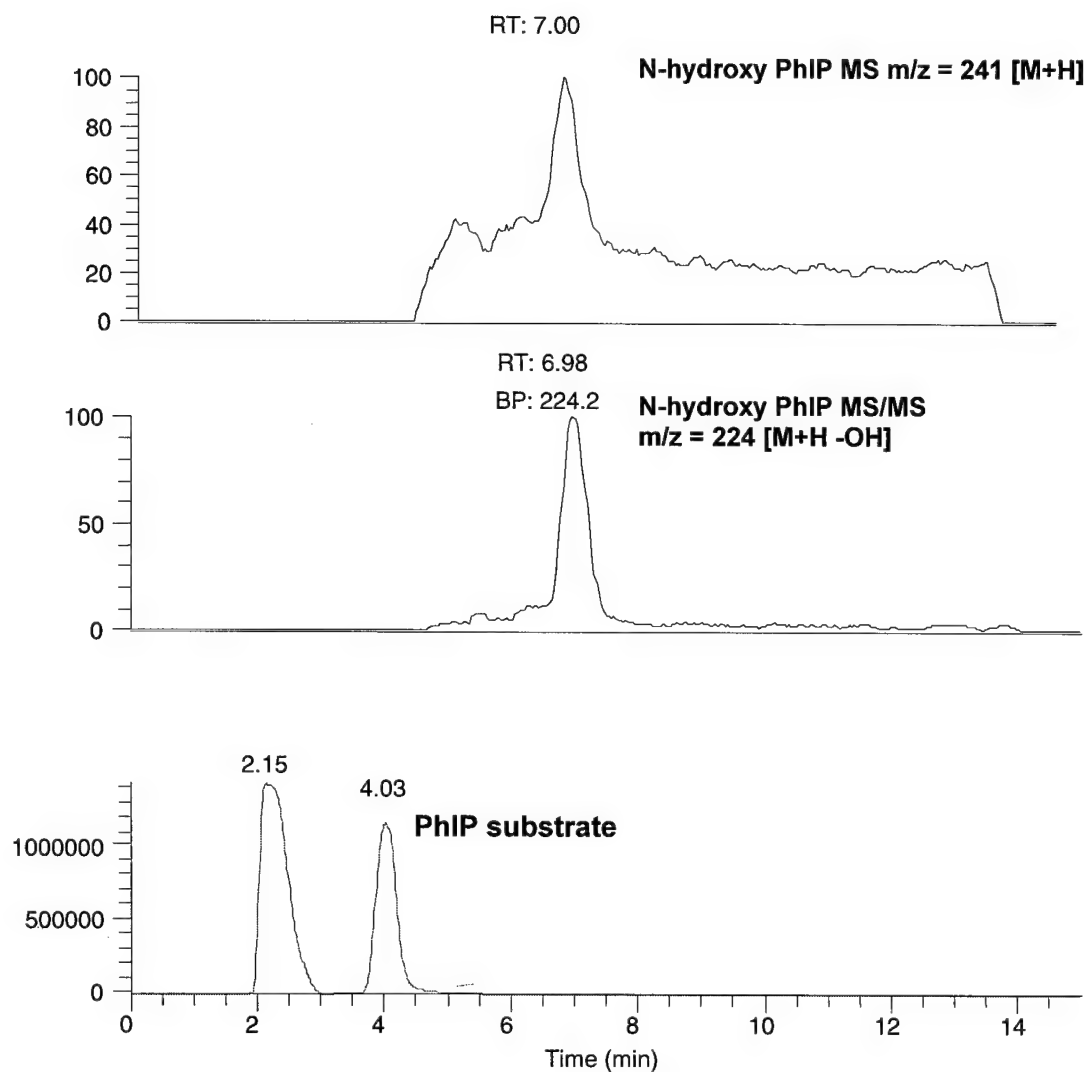


Figure 6: HPLC-UV, MS and MS/MS analysis of PhIP and *N*-hydroxy PhIP formation from the metabolism of PhIP by human prostate microsome P7 (140  $\mu$ g) incubated with 200  $\mu$ M of PhIP for 30 min.

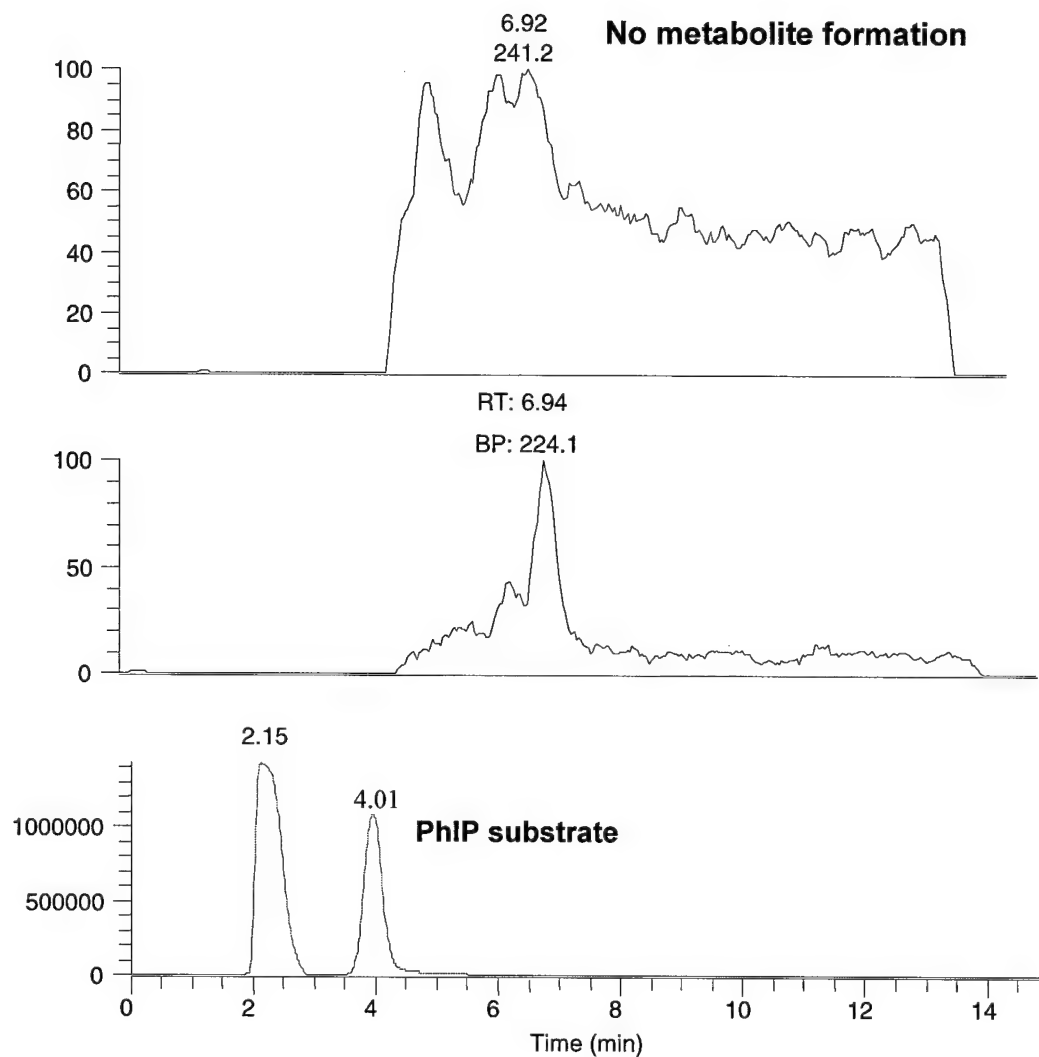


Figure 7: HPLC-UV, MS and MS/MS analysis of PhIP and *N*-hydroxy PhIP in the negative control. human prostate microsome 140  $\mu$ g denatured by ice cold methanol prior to incubation and incubated with 200  $\mu$ M of PhIP for 30 min. (Due to the very high sensitivity, the blank and negative control readings obtained in the MS/MS mode were subtracted for base line correction.)

RT: 0.00 - 2198 SM: 7B

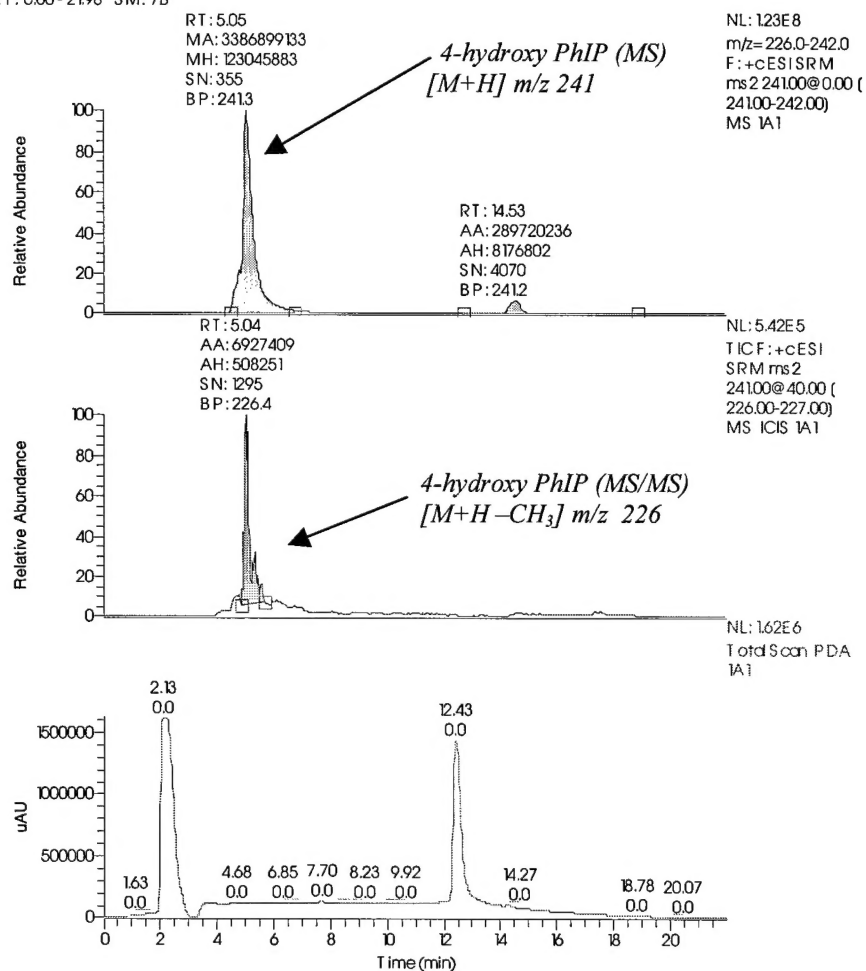
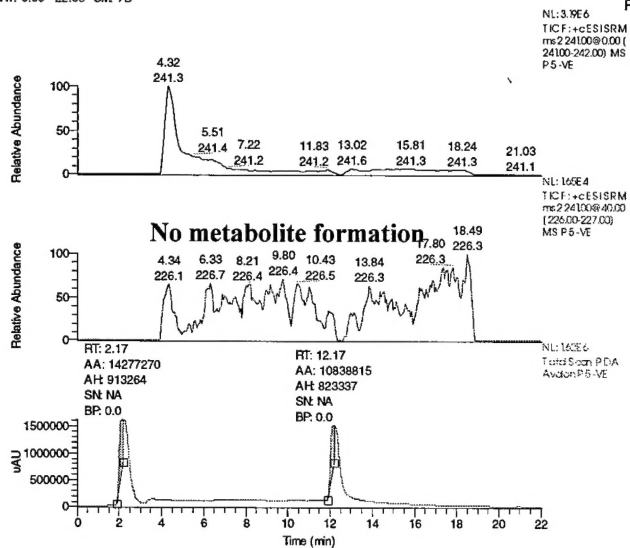


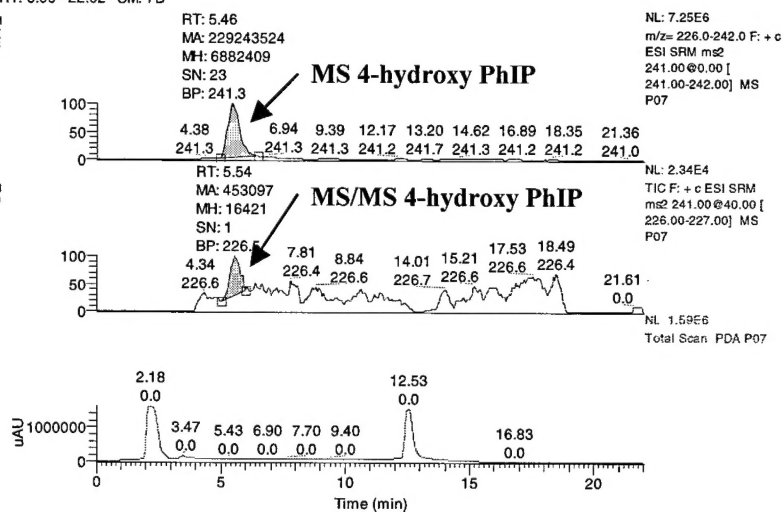
Figure 8: HPLC-UV, MS and MS/MS analysis of PhIP and 4-hydroxy PhIP, metabolism of PhIP by 5 pmol of human recombinant CYP1A1 incubated with 200  $\mu$ M PhIP for 30 min.

RT: 0.00 - 22.03 SM: 7B



(a)

RT: 0.00 - 22.02 SM: 7B



(b)

Figure 9: HPLC-UV, MS and MS/MS analysis of PhIP and 4-hydroxy PhIP, metabolism of PhIP by human prostate microsome incubated with 200  $\mu$ M PhIP for 30 min. (a) negative control where the microsomes were denatured prior to incubation and (b) is incubation with prostate microsome P7.



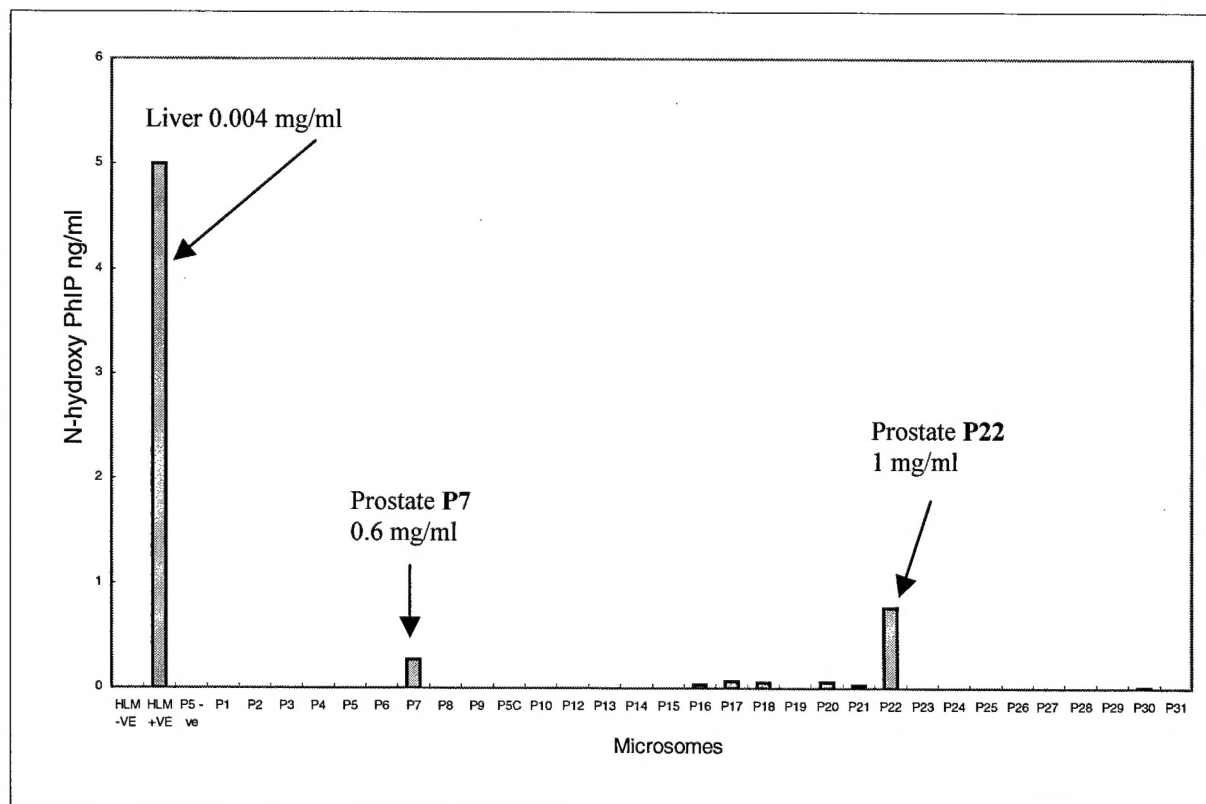


Figure 10: Comparison of metabolic activation of PhIP in human liver and prostate microsomes, final protein concentration was 0.004 mg/ml in human liver microsomes and in case of prostate P7 and P22 the protein concentration was 0.6 mg/ml and 1 mg/ml respectively.

**Table 1. Demographic data of subjects**

<b>ID</b>	<b>Age</b>	<b>Ethnic Status</b>	<b>Cause of surgery</b>
P0	85	Caucasian	BPH
P1	73	Caucasian	BPH
P2	61	Caucasian	BPH & Bladder cancer
P3	76	Caucasian	BPH & prostate cancer
P4	77	Caucasian	BPH & Bladder cancer
P5	67	Caucasian	Chronic Prostatitis & BPH
P5c	67	Caucasian	Chronic Prostatitis & BPH
P6	54	Not available	Normal / unknown
P7	54	Not available	Normal prostate/ Bladder cancer
P8	81	Not available	BPH
P9	64	Not available	BPH
P10	75	Caucasian	BPH
P12	69	Caucasian	BPH
P13	68	Black	Chronic Prostatitis & BPH
P14	63	Caucasian	BPH
P15	72	Black	BPH
P16	66	Caucasian	BPH & prostate cancer
P17	75	Caucasian	BPH & prostate cancer
P18	68	Caucasian	BPH & prostate cancer
P19	71	Caucasian	BPH & prostate cancer
P20	75	Caucasian	Chronic Prostatitis & BPH
P21	77	Caucasian	BPH
P22	60	Caucasian	Normal prostate/ Bladder cancer
P23	66	Hispanic	BPH & prostate cancer
P24	77	Caucasian	Chronic Prostatitis & BPH
P25	62	Black	BPH
P26	60	Caucasian	Chronic Prostatitis & BPH
P27	75	Caucasian	BPH
P28	59	Black	BPH
P29	57	Caucasian	BPH
P30	58	Caucasian	BPH
P31	76	Caucasian	BPH

*(BPH, Benign Prostatic Hyperplasia)*